5. MATERIALS AND METHODS

5.1. SAMPLING

In the present study the abiotic (Water and Sediment) and biotic pond snail (Lymnaea sp.), six species fish; (Oreochromis mossambicus (Tilapia), Channa striata (Snakehead murrel), Bagarius sp. (Cat fish), Anabas testudineus (Climbing perch), Trichogaster trichopterus (three spotted Gourami) and Glossogobius giuris (Tank gopy), were selected at random from the catch. These fish species are commonly consumed by the local communities. Fish identification, mass and fish total length were recorded before portioning the samples. The other samples were also collected such as Philateus sp. (Frog), Corvus corax (Crow), Egretta garzetta (Little Egret) and Calotes versicolor (Garden Lizard) and Human breast milk samples were collected in and around the Pallikaranai swamp during January 2013. The method of extraction and quantification of the organochlorine pesticides were given below.

5.2. THE ANALYTICAL PROCESS

The development of a complete analytical method includes a number of steps from sample collection to data handling. The intermediate steps of a complete analytical process include sampling, sample preparation, separation, detection, identification and quantification of the target compounds.
Stages of a complete analytical process

- **Sampling**
  - Collection
  - Storage

- **Sample Preparation**
  - Sample Work-up
  - Reduction of particle size
  - Removal of moisture
  - Extraction & Derivatization
  - Clean-up & Concentration

- **Instrumental Analysis**
  - Separation
  - Identification
  - Quantification

- **Report**
  - Results
Photo - 3. Collection of water sample from swamp

Photo - 4. Collection of sediment sample from Swamp
5.2.1. Extraction of pesticide from water samples:


Extraction, purification of extracts and analysis were done according to the method described for Water Analysis Method: 525.2 by GC/MS.

One litre water sample is collected from the Pallikaranai swamp during January 2013 using clean (washed and rinsed with Acetone & Hexane) amber glass bottles from wetlands in the Pallikaranai (Figure 2 & Figure 3) and transported on ice to the laboratory where they were stored at -20°C until extraction.

Hydrochloric acid is used at the sampling site to retard the microbiological degradation of some analytes in water. The sample pH is adjusted to <2 with 6 N hydrochloric acid. This is the same pH used in the extraction, and is required to support the recovery of acidic compounds like pentachlorophenol.

Within 48 hours the samples were filtered (1 μm GFC filters) and extracted onto C18 Liquid-Solid Extraction (LSE) Cartridges (6ml volume & 1gm sorbent weight) from Agilent Technologies (Agilent Bond Elut C18 LSE Cartridge).

Conditioning of each cartridge was done by eluting with a 5 mL aliquot of ethyl acetate followed by a 5 mL aliquot of methylene chloride. Cartridge was drained dry after each flush. Then cartridge was eluted with a 10 mL aliquot of methanol, and did not allow the methanol to elute below the top of the cartridge packing. From this point, the cartridge was not allowed go dry. 10 mL of reagent water was added to the cartridge and before the reagent water level drops below
the top edge of the packing. One litre of sample water was allowed to pass through the cartridge. This was completed in about two hours with the assistance of a slight vacuum of about 13 cm (5 in.).

One litre of water sample was poured into the 2 L separatory funnel with the stopcock closed, 5 mL methanol was added and mixed well. Periodically transfered a portion of the sample into the solvent reservoir. The water sample will drain into the cartridge, and from the exit into the suction flask. Maintaining the packing material in the cartridge immersed in water at all times. After all of the sample has passed through the PSE cartridge, draw air or nitrogen through the cartridge for 10 minutes.

Transfered the 125 mL solvent reservoir and PSE cartridge to the elution apparatus. The same 125 mL solvent reservoir is used for both apparatus. Rinsed the inside of the 2 L separatory funnel and the sample jar with 5 mL of ethyl acetate and eluted the cartridge with this rinse into the collection tube. Washed the inside of the separatory funnel and the sample jar with 5 mL methylene chloride and eluted the cartridge, collecting the rinse in the same collection tube. The eluate was passed through the drying column which is packed with approximately 5-7 g of anhydrous sodium sulfate and collected in a second vial. Washed with sodium sulphate and 2 mL methylene chloride and collected in the same vial. Concentrated the extract in a warm water bath under a gentle stream of nitrogen and made the concentrate to 1 mL.
All the solvents used in residue analysis were of analytical grade and thoroughly distilled in glass apparatus to remove trace impurities. All the glass wares were cleaned with Labolene (detergent) and soaked in concentrated sulphuric acid overnight. After thoroughly washing the glass wares in distilled water and subsequently with pesticide – free (hexane washed) water, they were air-dried and wrapped with pre-cleaned aluminium foil and baked in a hot air oven at 150°C overnight to remove traces of organic contaminant. They were rinsed with distilled n-hexane before use.

5.2.2. Extraction of pesticide residues in the sediment samples


Extraction, purification of extracts and analysis were done according to the method described for sediment analysis method USEPA -3540C.

Methods followed by Ramesh et al. (1991) for the collection and preservation and analysis of chlorinated pesticides in sediments was followed surficial sediment samples were collected at a water depth of 1.5 to 2 meter using a Teflon core sampler and the upper 5 cm sediment was used for analysis. Before analysis, the samples were air dried and ground in a pre-cleaned glass mortar and pestle and passed through a sieve (500 µm mesh size). Blended 10 g of the solid sample with 10g of anhydrous sodium sulfate and place in an extraction thimble. Placed approximately 300 mL of the extraction solvent (1:1 Acetone/Hexane into a 500-mL round bottom flask containing two clean boiling chips. The flask was attached to the extractor and extracted the sample for 16hrs.
Dried the extract by passing it through a drying column containing about 10 cm of anhydrous sodium sulfate. Concentrated the dried extract using a gentle stream of clean, dry nitrogen. The extracts obtained are analyzed for the target analytes using GCMS.

5.3. EXTRACTION OF PESTICIDE RESIDUES IN THE BIOLOGICAL SAMPLES

5.3.1. Tissues (Snail, fishes, frog, garden lizard, birds) and Milk Analysis method – AOAC 2007.01

Extraction, purification of extracts and analysis were done according to the method described for tissue and milk analysis in AOAC 2007.01

Organochlorine pesticides [HCH, (BCH) isomers (α, β, γ and δ), DDT and as metabolites (pp’ DDE, pp’ DDD, op’ DDT and pp’ DDT)] were analysed by following the methods of Tanabe et al. (1984) and Ramesh et al. (1992), with minor modifications.

Procedure:

Comminuted 1 kg sample with vertical cutter and Homogenized, 200 g subsample with probe blender. Twenty gram of the preserved tissue samples of snail, fishes, frog, garden lizard and birds were homogenized with anhydrous sodium sulphate to remove moisture. Transferred 15g of homogenized sample (or 15ml milk) to 50 mL Tetlon tube. 15 mL of 1%Hac in MeCN + 1.5g anh. NaAc+ 6g anhydrous MgSO₄ were added in to the tube was thoroughly shaken vigorously for 1 min. and centrifuged at >1500 rcf for 1 min. 2 ml oft this mixture was
transfered to tube. In which, 300 mg anhydrous MgSO$_4$ + 100 mg PSA + 100mg C18 were added and was shaking for 30 seconds. After this, the content was centrifuged at 1500 rcf for 1 minute.

During fractionation, the first fraction eluted with n-hexane contained pp’ DDE and the second fraction eluted with a 20% mixture of dichloromethane in n-hexane contained the HCH isomers. pp’ DDD, op’ DDT and pp’ DDT. Each fraction was concentrated, cleaned with sulphuric acid to remove interfering substances and then washed with hexane – washed water. 1 ml of the centrifuged extract was transferred to GC vial and analysed by GC-MS.

**5.4. QUANTIFICATION OF ORGANOCHLORINES**

Quantification of Pesticides  (alpha HCH, beta HCH) Lindane, op’ DDE, pp’ DDE, pp’ DDD, op’ DDT, were analysed by gas chromatography- mass spectrometry (GC-MS, Agilent GC 7890A with 7000 GCMS Triple Quad) according to USEPA standard methods (USEPA Method 8260C) after some modification. Target compounds were separated on a capillary column (HP-5MS, 30m × 0.25mm, 0.25 μm thick stationary phase) with helium as the carrier gas at a constant flow rate of 1.1 mL/min. Quantitative mass spectrometric analyses were conducted by using electron ionisation source (EI) and selected ion monitoring mode (SIM).

**5.4.1. Operating Conditions of the GC**

The injector and detector temperatures were 275 and 300°C respectively. Column temperature was 190°C held for 12 minutes and programmed at a rate of 5°C/min to 216°C and held for 20 minutes. Nitrogen (IOLAR Grade I) was used
as the carrier gas at a flow rate of 30 ml/minute. Standard mixtures and fortified samples were frequently used to check the accuracy of quantification. Organochlorine pesticides were qualified as individually resolved peaks based on retention times, in comparison with the corresponding peak heights of standards (Ultra Scientific Co., USA). The detection limit of the analysed organochlorine compounds was 0.01 ng g\(^{-1}\) for all the samples. The recovery efficiency of HCH isomers and DDT compounds varied between 89 and 92 percent. Results were not corrected for recovery percentage.